DEVELOPMENT AND VALIDATION OF VIRUS ISOLATION FOR RABIES DIAGNOSIS DURING OUTBREAK IN SARAWAK

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ABSTRACT. Disadvantages of using FAT in rabies diagnosis include the inability of the test to be performed on large number of samples that will possibly produce false negative results. Hence, a virus isolation test (VI) using tissue culture technique has been developed and validated for routine rabies diagnosis, used alongside fluorescence antibody test (FAT). Field cases were submitted during rabies outbreak in Malaysia between 2017 until 2019. The test was established in 4-wells chamber slide with confluence Murine Neuroblastoma Cells (N2a). The brain suspension of suspected rabies cases was inoculated and incubated at 37 ℃ for 96 hours. The cultures were then fixed using acetone and stained with fluorescein-labelled rabbit antibody-conjugate, then examined under fluorescence microscope. Of the 224 brain samples tested, 52 (23.21 %) were positive by VI but 2 (0.89 %) were negative by FAT, whereas 126 (56.25 %) were negative by VI but 44 (19.55 %) were positive by FAT. The sensitivity and specificity of the VI against FAT were 54.17 % and 98.44 % respectively. The VI appeared to be reliable to detect live viruses and can be served as a confirmation test against FAT.

Keywords: rabies, FAT, virus isolation, rapid test

INTRODUCTION

Rabies is a fatal disease that can present as classic furious rabies or paralytic rabies (Hemachudha *et al.*, 2013). The disease causes 50,000 to 55,000 human deaths each year, with 25,000-30,000 human deaths in India alone. There are over 3 billion people continuing to be at risk of rabies in over 100 countries in the 21st century (Wunner & Briggs, 2010). In 2015, a small outbreak occurred in the northern states of Peninsular Malaysia, namely Perlis, Kedah, Kelantan, and Selangor (Muthu, 2018) followed by a rabies outbreak in Sarawak involving humans and animals (Navanithakumar *et al.*, 2019).

The Veterinary Research Institute (VRI) serves as the reference laboratory for rabies diagnosis in Malaysia. As a reference laboratory, effective measures and accurate results must be provided to control the disease and subsequently, to regain rabies-free status in Malaysia (Navanithakumar *et al.*, 2019).

Fluorescence antibody test (FAT) is the main diagnostic method for rabies as it is the gold standard technique as recommended by OIE and WHO. The test has been proven to be fast and sensitive to detect rabies antigen in brain samples and salivary glands of rabid animals (Goldwasser *et al.*, 1959). However, drawbacks of FAT include inability to be performed with ease onto large number of samples (Bourhy *et al.*, 1989) and can result in false-positive result if proper sample preservation was not applied (Nwosuh *et al.*, 2009).

Hence, virus isolation (VI) of rabies virus has been introduced as alternative technique

for rabies diagnosis to be used alongside FAT. Many studies have shown that street rabies virus can grow well into tissue culture. Larghi (1975) developed a tissue culture system using BHK-21 cells supplemented with Diethylaminoethyl-Dextran to increase sensitivity for detection of street rabies virus in saliva samples. A few years later, Webster (1987) performed isolation of the virus using Murine Neuroblastoma Cells (Na2) inoculated with rabies suspected dog brain tissue in comparison to BHK-21. The Na2 cell has been proved to be highly sensitive to detect the virus compared to BHK-21. The following comparison study later done by Rudd *et al.* (1987) strengthen the fact that Na2 cells are more sensitive to street rabies virus. Thus, in view of the outbreak in Sarawak resulting in a dramatic increase of sample submission for rabies diagnosis, fast and accurate test was needed, which prompted the researchers to develop and validate rabies virus isolation using tissue culture technique with Na2 cells as an alternative test for diagnosing rabies.

MATERIALS AND METHODS

Samples

A total of 224 dog samples from surveillance area of Sarawak were submitted to VRI between September 2017 until January 2019. All specimens were transported using air transportation (MAS Cargo) and kept at 4-8 °C in the icebox. Upon arrival, the samples were processed and stored accordingly prior to diagnostic workup.

Fluorescence Antibody Test (FAT)

The FAT was performed according to Rabies OIE manual with the following modifications. The cerebrum, cerebellum, hippocampus, and brain stem of the dogs were smeared on test slide and then air dried. The slides were fixed with ice cold acetone for 30 minutes at room temperature before staining with a commercially available fluorescein-tagged monoclonal antibody conjugate (Fujirebio Diagnostic Inc, USA). Next, it was washed with phosphate-buffered saline (pH 7.6) for 3 minutes followed by washing with distilled water for 2 minutes. Finally, the test slide was mounted using fluorescence mounting medium (Dako, North America, Inc.) and examined under a fluorescence microscope using 20X and 40X magnifications.

Brain Suspension

The 10 % brain suspensions were prepared in Viral Transport media (VTM) supplemented with 5 % fetal bovine serum contained 200 IU/mL penicillin, 200 µg/mL streptomycin, 0.50 µg/mL amphotericin B, 200 µg/mL kanamycin sulphate (Gibco, USA), and 7.5 % of sodium bicarbonate. The brains were ground in a mortar and pestle, before being centrifuged at 3000 rpm for 10 minutes at 4 °C. A supernatant of centrifuged brain tissue was collected, diluted in 1:10 and was used as tissue culture infection inoculum.

Cell Culture

The Murine neuroblastoma cells (N2a) CCL-131™ in the American Type Culture Collection (ATCC®) were maintained in Eagle's minimum essential medium (EMEM) (Gibco, USA) supplemented with heat-inactivated 5 % fetal bovine serum (Gibco, USA) contained 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 100 µg/mL kanamycin sulphate (Gibco, USA) and 7.5 % of sodium bicarbonate. The cells were incubated at 37 °C in a moist atmosphere containing 5 % CO_2.

Prior to use in the tissue culture infection, the cells were trypsinized with Tryp-LE Express (Gibco, USA) and resuspended in EMEM contained 10 % heat-inactivated fetal bovine

serum (Gibco, USA) contained 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 100 µg/mL kanamycin sulphate (Gibco, USA) and 7.5 % of sodium bicarbonate. The cells were cultured in a concentration of 5 x 10^5 cells/mL in four-chamber slides (Nalgene Nunc International, Lab-Tek® II) and kept at 37 °C in a moist atmosphere containing 5 % CO_2.

Tissue Culture Infection

To each well, 0.1 mL of brain tissue suspension was added. The cultures were incubated 96 hours at 37 °C with presence of 5 % CO_2. Following incubation, the mediums were removed, and the wells were fixed with 80 % cold acetone for 30 minutes at room temperature. After fixation with acetone, the cultures were stained and incubated with FITC Anti-Rabies Monoclonal Globulin conjugate (Fujirebio) for 30 minutes at 37 °C. The cultures then were washed with phosphate-buffer saline (pH 7.2-7.4) for two times, 2 minutes each. The stained cultures were examined using fluorescence microscope under 20X and 40X objective.

Calculation of Sensitivity and Specificity

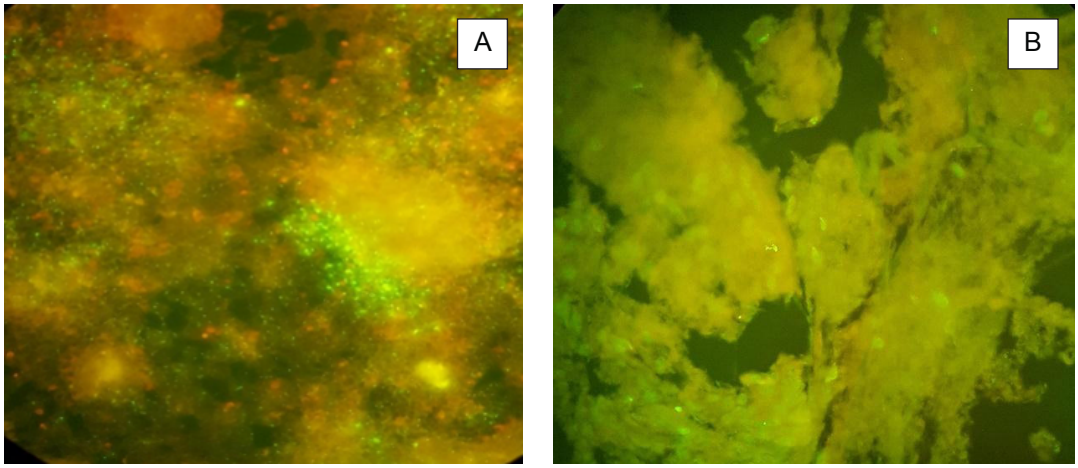
Sensitivity is calculated using formula [TP/(TP+FN)] x 100 where TP is the number of samples with true-positive results as determined by the reference assay and FN is the number of samples with false-negative result. Specificity is defined as [TN/(TN+FP)] x 100 where TN is the number of samples with true-negative results and FP is the number of samples with false-positive results (Parikh *et al.*, 2008).

RESULTS

The results obtained on 224 samples are summarized in Table 1. Of the 224 samples, 52 (23.21 %) were positive by VI but 2 (0.89 %) were negative by FAT, whereas 126 (75.89 %) were negative by VI but 44 (56.25 %) were positive by FAT. Table 1 also shows that the sensitivity and specificity of the VI were 54.17 % and 98.44 % respectively.

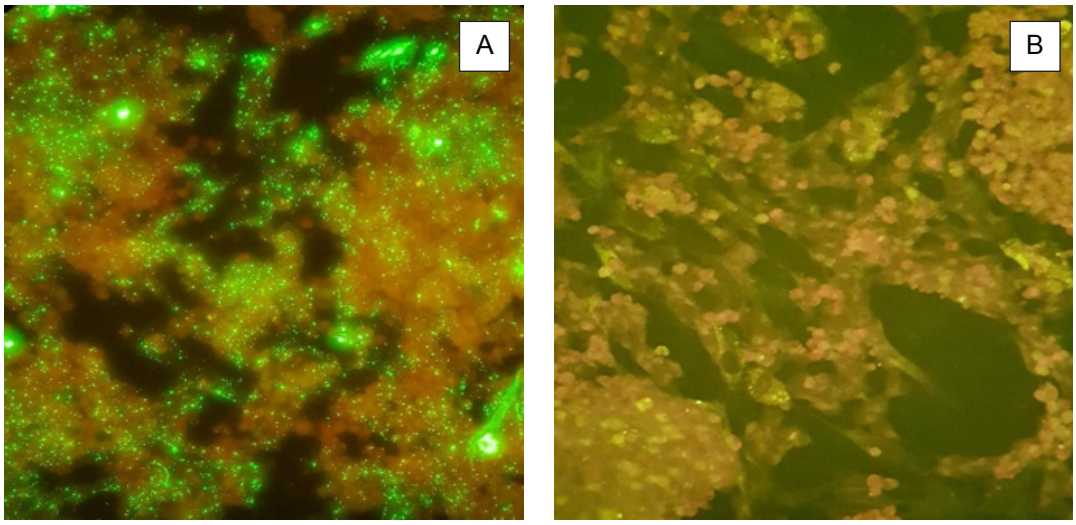
Table 1. Comparison between the results obtained by VI and FAT on 244 brain samples received at VRI during the outbreak for rabies diagnosis with specificity and sensitivity.

VI results	FAT results		
	Positive	Negative	Total
Positive	52	2	54
Negative	44	126	170
Total	96	128	224
Sensitivity (%)	54.17 %		
Specificity (%)		98.44 %	



(A) Positive dog brain sample (B) Negative dog brain sample

Figure 1. Appearance of rabies-specific fluorescent foci in the dog brain using x40 magnification. (A) Positive result of dog brain sample (B) negative result of dog brain sample.



(A) Positive result of VI (B) Negative result of VI

Figure 2. Appearance of rabies-specific fluorescent foci in the VI test using x20 magnification. (A) Positive result of VI (B) Negative result of VI

DISCUSSION

In 96 of 224 samples tested by FAT, 42.9 % were positive for rabies while 54 (24.1 %) were positive by VI. This indicated that the suspected animals were confirmed to be infected with rabies virus. However, the results showed that only 52/96 (54.2 %) of rabies samples that were positive by FAT showed bright apple green fluorescence in VI (Table 1, Figure 1). The lower positivity of the VI (24.1 %) compared to FAT (42.9 %) and the ability of the FAT to detect rabies antigen in 44 of the 170 samples that tested negative by VI may be caused by intolerance of tissue culture procedure with decomposed tissue. After days of decomposition, the brain tissue suspension was toxic for tissue culture growth. Poor sample condition resulted to the condition where N2a lysis and clumping produced unsatisfactory result whereas effect of degradation samples caused validity of rabies diagnosis (Albas *et al.*, 1999; David *et al.*, 2002; Lopes *et al.*, 2010). Other author also reported the same issue that tissue culture procedures is less tolerant to decomposed tissue than FAT (Rudd *et al.*, 1989) but rabies antigen still can be detected on decomposed tissue by FAT with reduced fluorescence intensity as reported by Rudd (1989).

The sensitivity of VI in comparison with FAT was 54.17 %, while the specificity was 98.44 %. This showed that VI missed rabies antigen in some positive samples in the study resulting in false negative result. However, the high specificity of the VI means that there were no false positive results from samples that were tested positive by the VI. Therefore, a positive result guarantees that the samples is truly positive while negative result does not. This further implies that the VI procedure is only useful when the test result of rabies-suspected animal is positive but doubtful when result is negative.

During this study, the researchers had to modify some of the procedures to accommodate the situation. This study shows that at 100 % of concentration of acetone, the well chambers of the slides detached due to the corrosiveness of acetone which melted the chamber adhesive. However, study by Anca (2017) showed that absolute acetone was well adapted on fixation immunofluorescence microscopy for rabies virus detection. Later in this study, the acetone with 70 % - 80 % concentration was used to prevent the chamber detachment and achieve better tissue fixation. In addition, distilled water was used to remove excess stains on fixed glass. The excess stain caused poor clarity during observation under fluorescence microscope.

Given that rabies is a deadly disease with 100 % case fatality rate once the virus has entered the central nervous system (CNS), the need to develop a highly sensitive, rapid, reliable, and standardize test for routine rabies diagnosis and during outbreak investigation, in order to facilitate decision-making regarding the possible treatment and eradication of the disease cannot be neglected. Rapid and reliable rabies detections can be a cost-effective approach to management of dog bite victims considering the high cost of rabies post-exposure treatment (Robles *et al.*, 1992). Any false negative results may give higher impact and false positive results can lead to unnecessary judgment (Yang *et al.*, 2012). A positive test by any standard procedures overrides negative reaction to each other. The inconclusive result obtained in any single test must be re-test again to achieve definite conclusion.

This development results show that the VI procedure are comparable, but the FAT is more superior test than VI when dealing with decomposed tissues. The VI result show

concordance with FAT. This is due to a large part of their specificity, which is 98.44 % slightly lower as reported by another author (Bourhy *et al.*, 1989). The lower sensitivity (54.17 %) corroborating that the use of VI instead of FAT is not recommended (Perrin' *et al.*, 1987). The procedure could be very useful to many laboratories that rely exclusively on FAT results or still use MIT as a backup procedure to corroborate FAT results. However, VI requires more expensive equipment than FAT, in addition to requiring personnel trained in cell culture.

Therefore, it is recommended to routinely use VI for confirmation diagnosis on rabies in suspected animals to facilitate decision-making when handling exposed humans.

CONCLUSION

This study findings support that the new established VI appears to be reliable for isolation of street rabies virus and serve as an alternative test to support FAT results. However, further studies are needed to confirm the practicality of the test on routine rabies diagnosis in Malaysia.

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